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Abstract: Sézary syndrome (SS) is a rare, leukemic type of cutaneous T-cell lymphoma (CTCL), for which extracorporeal photopheresis (ECP) is a first-line therapy. Reliable biomarkers to objectively monitor the response to ECP in patients with SS are missing. We examined the quantitative and qualitative impact of ECP on natural killer (NK) cell activity in SS patients, and especially their functional ability for antibody-dependent cell-mediated cytotoxicity (ADCC). Further, we addressed the question whether the magnitude of the effect on ADCC can be associated with the anti-cancer efficacy of ECP in SS patients. We assessed numbers of NK cells, ADCC activity, and treatment response based on blood tumor staging in a cohort of 13 SS patients (8 women, 5 men) treated with ECP as a first-line therapy. Blood samples were collected before treatment start and after an average of 9 months of uninterrupted ECP treatment. NK cell numbers were reduced in SS patients compared to healthy individuals and showed a tendency of recovery after long-term ECP treatment, independent of the clinical response to treatment. Patients with marginal increase (1.5 AU-fold) or lack of increase in ADCC activity failed to respond clinically to treatment, while patients with an increased ADCC activity showed a reduction in blood tumor burden. NK-mediated ADCC is selectively enhanced and might be a mechanism underlying the effect of ECP while in addition it can possibly serve as a reliable biomarker to objectively monitor response to ECP in patients with SS.

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ORIGINAL RESEARCH

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Enhancement of antibody-dependent cellular cytotoxicity is associated with treatment response to extracorporeal photopheresis in Sézary syndrome

Christoph Iselin^a, Yun-Tsan Chang^{a,b}, Tanja Schlaepfer^c, Christina Fassnacht^{a,d}, Florentia Dimitriou^a, Mirjam Nägeli^a, Steve Pascolo^a, Wolfram Hoetzenecker^d, Malgorzata Bobrowicz^d, and Emmanuella Guenova^{a,b}

^aDepartment of Dermatology, University Hospital Zurich, University of Zurich, Zurich, Switzerland; ^bDepartment of Dermatology, Lausanne University Hospital CHUV, University of Lausanne, Lausanne, Switzerland; ^cDepartment of Dermatology, Cantonal Hospital St. Gallen, St. Gallen, Switzerland; ^dDepartment of Dermatology, Kepler University Hospital, Linz, Austria; ^eDepartment of Dermatology, Lausanne University Hospital CHUV and Faculty of Biology and Medicine, Lausanne University, Lausanne, Switzerland

Summary

Sézary syndrome (SS) is a rare, leukemic type of cutaneous T-cell lymphoma (CTCL), for which extracorporeal photopheresis (ECP) is a first-line therapy. Reliable biomarkers to objectively monitor the response to ECP in patients with SS are missing. We examined the quantitative and qualitative impact of ECP on natural killer (NK) cell activity in SS patients, and especially their functional ability for antibody-dependent cell-mediated cytotoxicity (ADCC). Further, we addressed the question whether the magnitude of the effect on ADCC can be associated with the anti-cancer efficacy of ECP in SS patients. We assessed numbers of NK cells, ADCC activity, and treatment response based on blood tumor staging in a cohort of 13 SS patients (8 women, 5 men) treated with ECP as a first-line therapy. Blood samples were collected before treatment start and after an average of 9 months of uninterrupted ECP treatment. NK cell numbers were reduced in SS patients compared to healthy individuals and showed a tendency of recovery after long-term ECP treatment, independent of the clinical response to treatment. Patients with marginal increase (≤ 1.5 AU-fold) or lack of increase in ADCC activity failed to respond clinically to treatment, while patients with an increased ADCC activity showed a reduction in blood tumor burden. NK-mediated ADCC is selectively enhanced and might be a mechanism underlying the effect of ECP while in addition it can possibly serve as a reliable biomarker to objectively monitor response to ECP in patients with SS.

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Introduction

Sézary syndrome (SS) is a rare leukemic type of cutaneous T-cell lymphoma (CTCL) representing around 5% of all CTCL cases.^{1,2} SS typically manifests with a triad of erythroderma, generalized lymphadenopathy, and the presence of malignant T cells in the skin, lymph nodes, and peripheral blood, morphologically presenting with “cerebriform” nuclei.^{3,4} Numerous immune abnormalities have been described in SS patients, most commonly reported among those are the increased secretion of immunosuppressive Th2 cytokines and the decreased proinflammatory Th1-mediated immune response.^{5–7} These alterations lead to global immunosuppression and increased susceptibility to infections which are a leading cause of death in SS patients.⁸ Therefore, therapeutic approaches that aim at immune modulation are standard therapies in SS. Currently, extracorporeal photopheresis (ECP) and administration of interferon- α , are established and recommended first-line immunomodulatory treatment options in SS.¹ ECP is a leukapheresis-based therapy, where the patient’s lymphocytes are collected and exposed to methoxsalen followed by ultraviolet A light, before being reinfused to the patient. ECP is nowadays available in more than 200 centers worldwide with cited response rates for CTCL of

approximately 60% and complete responses of 14–26%.^{9,10} ECP is used as a monotherapy or in combination with other immunomodulatory agents, and is well-tolerated.^{10,11} Given the interpatient variability of response to ECP, the identification of factors associated with a positive response to ECP at baseline is of great interest.

In this work, we aimed to answer the question whether ECP influences the number of NK cells and the efficacy of NK-mediated antibody-dependent cellular cytotoxicity (ADCC). ADCC is an adaptive immune response, where Fc receptor-bearing NK cells recognize and kill antibody-coated target cells by releasing cytotoxic molecules, such as perforin and granzymes. Furthermore, we aimed to explore if the magnitude of ADCC can be associated with the treatment response in SS patients.

Materials and methods

Patients and collection of primary human samples

13 patients newly diagnosed with Sézary syndrome between 2012 and 2019 treated with ECP as a first-line systemic monotherapy with at least 1.5 months clinical follow-up after the start of the ECP therapy were included in a single-center,

retrospective, cohort study (Table 1). Diagnosis of Sézary syndrome was established according to the criteria of the World Health Organization–European Organization for Research and Treatment of Cancer and the International Society for Cutaneous Lymphomas.^{4,12} ECP treatment was performed using the closed photopheresis apparatus “CELLEX”

(Mallinckrodt Pharmaceuticals, USA). Treatment schedule followed the European dermatology forum-guidelines on the use of extracorporeal photopheresis, with one ECP cycle (on two consecutive days) every 2 weeks for the first 3 months, followed by one ECP cycle every 4 weeks.⁹ The peripheral blood samples were collected during routine ECP procedure right before the

Table 1. Clinical patients' characteristics.

Pat.No. FACS Figure 1	Pat.No. ADCC Figure 2	Diagnosis	Disease stage	Sex (female/ male)	Staging parameter	Before ECP	After ECP	Overall response	Responder (R)/ Non- Responder (N)	ECP stopped/continued
1	1	SS	stage IVA1 T4N1M0B2	F	CD4:CD8 CD4+ CD26-/CD7- (cells/ μ l) CD4+ CD26-/CD7- (%) Blood stage	47.5 6605 97 B2	2.1 158 40 B0	CR	R	stopped due to remission
2	-	SS	stage IVA1 T4N1bM0B2	F	CD4:CD8 CD4+ CD26-/CD7- (cells/ μ l) CD4+ CD26-/CD7- (%) Blood stage	82 582 51 B1	93 1202 45 B2	SD	N	stopped due to death
3	3	SS	stage IVA1 T4N0M0B2	F	CD4:CD8 CD4+ CD26-/CD7- (cells/ μ l) CD4+ CD26-/CD7- (%) Blood stage	47.5 9790 93 B2	47.5 4736 44 B2	PR	R	stopped due to patients' wish
4	4	SS	stage IVA1 T4NxM0B2	M	CD4:CD8 CD4+ CD26-/CD7- (cells/ μ l) CD4+ CD26-/CD7- (%) Blood stage	15.2 4657 72 B2	8.4 1243 73 B2	PR	R	stopped due to inclusion in clinical trial
5	5	SS	stage IVA1 T4NxM0B	F	CD4:CD8 CD4+ CD26-/CD7- (cells/ μ l) CD4+ CD26-/CD7- (%) Blood stage	48 11135 94 B2	47.5 5939 94 B2	SD	N	stopped due to lost to follow up
6	6	SS	stage IVA1 T4NxM0B	F	CD4:CD8 CD4+ CD26-/CD7- (cells/ μ l) CD4+ CD26-/CD7- (%) Blood stage	4.6 989 49 B1	8.2 1540 81 B2	SD	N	continued (since 4.5 years)
7	7	SS	stage IVA1 T4NxM0B2	F	CD4:CD8 CD4+ CD26-/CD7- (cells/ μ l) CD4+ CD26-/CD7- (%) Blood stage	1.1 346 57 B1	1.9 381 64 B1	SD	N	stopped due to lost to follow up
8	8	SS	stage IVA1 T4NxM0B2	F	CD4:CD8 CD4+ CD26-/CD7- (cells/ μ l) CD4+ CD26-/CD7- (%) Blood stage	44.2 3556 92 B2	31 5424 96 B2	SD	N	stopped due to inclusion in clinical trial
9	9	SS	stage IVA1 T4N0M0B2	M	CD4:CD8 CD4+ CD26-/CD7- (cells/ μ l) CD4+ CD26-/CD7- (%) Blood stage	6.8 1529 78 B2	5 226 39 B0	CR	R	continued (since 1.5 years)
-	10	SS	stage IVA1 T4N1M0B2	F	CD4:CD8 CD4+ CD26-/CD7- (cells/ μ l) CD4+ CD26-/CD7- (%) Blood stage	10.4 1923 77 B2	48.5 3876 86 B2	SD	N	continued, plus additional therapy with MTX
-	11	SS	stage IVA1 T4N1M0B2	M	CD4:CD8 CD4+ CD26-/CD7- (cells/ μ l) CD4+ CD26-/CD7- (%) Blood stage	14.3 2356 91 B2	7.3 541 59 B1	PR	R	continued (since 4 years)
-	12	SS	stage IVA2 T4N3M0B2	M	CD4:CD8 CD4+ CD26-/CD7- (cells/ μ l) CD4+ CD26-/CD7- (%) Blood stage	1.5 900 73 B1	1.2 1451 64 B2	SD	N	continued (since 3 years)
-	13	SS	stage IVA1 T4N0M0B2	M	CD4:CD8 CD4+ CD26-/CD7- (cells/ μ l) CD4+ CD26-/CD7- (%) Blood stage	1.1 133 46 B0	2 90 61 B0	SD	N	stopped due to death

MTX: Methotrexate

Responder: CR, PR

Non-responder: SD, PD

SS: Sézary Syndrome

ECP: Extracorporeal photopheresis

CR: complete response

PR: partial response

SD: stable disease

PD: progressive disease

initiation of ECP treatment and on average after 9 months of uninterrupted ECP treatment. In detail, 69.2% (9/13) of the baseline pre-ECP samples were collected within 2 days before ECP initiation, 30.7% (4/13) samples were collected at a median of 2.5 months (range 1–7) before therapy start. 61.5% (8/13) of the post-ECP samples were collected after a median of 6.75 months (range 1.5–9 months), and 38.5% of the samples were from patients on long-term ECP and were collected after a median of 13 months (range 12–26 months). Blood samples from healthy individuals served as control where indicated. Peripheral blood from the patients was collected in the context of the University of Zürich Biobank, funded by the University of Zurich University Research Priority Program (URPP) in translational cancer biology. All patients signed an informed consent agreeing to the use samples, including the generation of cell cultures according to the Biobank project (EK No. 647). Blood from healthy individuals was obtained anonymously from the blood bank of the University Hospital Zurich. The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of the Canton of Zurich (KEK-ZH-No. 2015–0209).

Clinical evaluation and response criteria

Assessment of clinical response to treatment was done according to the consensus statement of the International Society for Cutaneous Lymphomas (ISCL), the United States cutaneous lymphoma consortium, and the cutaneous lymphoma task force of the European Organization for Research and Treatment of Cancer (EORTC).¹³ Briefly, patients that reached B0 under therapy were considered in complete remission (CR). Partial remission (PR) was defined as >50% decrease in quantitative measurements of blood tumor burden from baseline in those with high tumor burden at baseline (B₂). Progressive disease (PD) was defined as > 50% increase from baseline and at least 5,000 neoplastic cells/ μ l blood. Stable disease (SD) includes patients that fail to attain criteria for CR, PR, or PD. For the current analysis, patients with CR or PR were considered “responders” and patients with SD or PD as “non-responders” to ECP treatment.

Isolation of human peripheral blood mononuclear cells (PBMCs) and cell lines

Patient's peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation. Raji, a Burkitt's lymphoma cell line was a kind gift from Prof. Magdalena Winiarska, Department of Immunology, Medical University of Warsaw.

Flow cytometry

To assess the percentage of NK cells in the collected PBMCs, the samples were analyzed by flow cytometry using anti-human monoclonal antibodies: CD3 (clone BW264/56, PerCP; Miltenyi Biotec #130-096-910), CD4 (Clone VIT4; label APC-Vio770; Miltenyi Biotec # 130–098-153), CD8 (clone RPA-T8, PE; Beckton Dickinson) and CD56 (clone AF12-7H3, PE-Vio770; Miltenyi Biotec #130-096-831). Samples were acquired

on Becton Dickinson, Allschwil, Switzerland FACSCanto II instrument. Flowjo V 10.0.8r1, Becton Dickinson and Company, Franklin Lakes, NJ, USA, Origin Pro 2017, Northampton, MA, USA and GraphPad Prism 5.0 Software, San Diego, CA, USA were used for data analysis and statistics.

Antibody-dependent cellular cytotoxicity assay

To assess NK cell activity in primary human samples, we resuspended the PBMCs in 5 ml cRPMI 1640 supplemented with 100 IU/ml IL-2 (manufactured by ourselves using human-IL-2-transfected J558 cells) and 10 ng/ml IL-15 (#200-15 Peprotech, Rocker Hill, USA) and incubated them on 6-well plates for 24 h (37°C, 5% CO₂). After 24 hours, the samples were centrifuged (1200 rpm, 3 min RT), resuspended with 1 mL RPMI 1640, counted, adjusted to a concentration of 4×10^6 cells/ml and used in ADCC assay as effector PBMCs. For the ADCC assay, cells from the CD20-expressing Raji cell line, maintained in cRPMI at 37°C, 5% CO₂, were adjusted to a final concentration of 2×10^5 cells/ml and 25 μ l of the Raji cell-containing solution was seeded on a 96 well plate. 25 μ l of the anti-human CD20 monoclonal antibody Rituximab (RTX, #B6097, Roche, Basel, CH) at a concentration of 300 μ g/ml (Raji 100), 30 μ g/ml (Raji 10) or 0 μ g/ml (Raji 0) was added and the cells were incubated in 37°C for 15 min to enable surface labeling with the antibody. Finally, 25 μ l of the effector PBMCs (4×10^6 cells/ml) were added, the plates were centrifuged (3 min, 750 rpm, RT) and incubated at 37°C, 5% CO₂ for 4 hours. The cell lysis was quantified by the amount of lactate dehydrogenase (LDH) released into the culture supernatant and measured with a commercially available kit according to the manufacturer's instructions (Pierce™ LDH Cytotoxicity Assay Kit; 88953, ThermoFisher, Waltham, USA). Maximum LDH release was determined by lysis in 0.2% Triton X-100. All experiments were performed in triplicates. LDH release was detected at 490 nm (LDH measurement) and 630 nm (background) using a microplate reader (ELx808 Absorbance Reader; BioTek; Winooski, USA). The effective ADCC in arbitrary units (AU) was calculated as the ratio between LDH absorption in the presence of 100 μ g/ml RTX and in the absence of RTX [ADCC [AU] =].

Statistical analysis

Data were tested for normal distribution with Shapiro-Wilk test. Dependent on the distribution pattern, Mann Whitney test for unpaired, not normally distributed sample groups and Wilcoxon matched pairs signed rank test for paired, not normally distributed sample groups. *P* values ≤ 0.05 were considered significant. Statistical calculations were done on GraphPad Prism® 7.01 software.

Results

NK cell numbers are reduced in SS patients compared to healthy individuals

In graft-versus-host disease (GvHD) patients for whom ECP therapy is commonly used, ECP treatment affects the NK cell

compartment,¹⁴ and an increase of the CD56^{bright} NK subset is predictive of response to treatment.¹⁵ To study the role of NK cells in the response in SS patients, we first assessed NK cell number in peripheral blood of SS patients compared to healthy individuals by flow cytometry. At baseline, we detected a significant decrease in CD3⁺ and CD56⁺ NK cell numbers (gating strategy shown in Figure 1a) in SS patients with a median of 92 cell/ μ l as compared to healthy individuals with a median of 238 cell/ μ l (p value: 0.00045) (Figure 1b). To study the effect of ECP, we compared the NK cell numbers at initiation of ECP treatment and after an average of 9 months of uninterrupted ECP in a cohort of nine SS patients (Table 1). Following ECP, we detected a slight increase of total CD56⁺ NK cells (Figure 1c), as well as of the CD56^{dim} (Figure 1d) and CD56^{bright} (Figure 1e) NK cell subsets with great interpatient variability. Although we observed a general tendency of recovery of diminished NK cell subsets in SS patients on long-term ECP treatment, none of the reported quantitative changes reached statistical significance or could be associated with the treatment response (Figure 1c-f).

ADCC is enhanced in ECP responders

The quality of the NK cell activity might be a positive response predictor for ECP.¹⁶ As antibody-dependent cellular cytotoxicity (ADCC) is a major effector function of NK cells,¹⁷ we evaluated its efficacy in SS patients before and after long-term ECP treatment. While a standard LDH release assay revealed no significant difference in the ADCC efficacy in SS patients at baseline (mean 1.087 AU) compared to healthy individuals (mean 1.25 AU) (Figure 2a), there was a significant increase in the ADCC efficacy in SS patients upon long-term uninterrupted ECP treatment (mean 2.28 AU ($p = .004$)) (Figure 2a). In general, at a threshold of ≥ 1.5 AU-fold, the efficacy of ADCC was increased in 41.66% (5/12) of all ECP treated SS patients (Figure 2b). When stratified according to their clinical response to ECP treatment, all but one of the patients with increased ADCC upon treatment ≥ 1.5 AU-fold responded clinically, while patients with marginal increase (≤ 1.3 AU-fold) or lack of increase in ADCC activity failed to respond to ECP treatment (Figure 2c). Furthermore, the overall level of

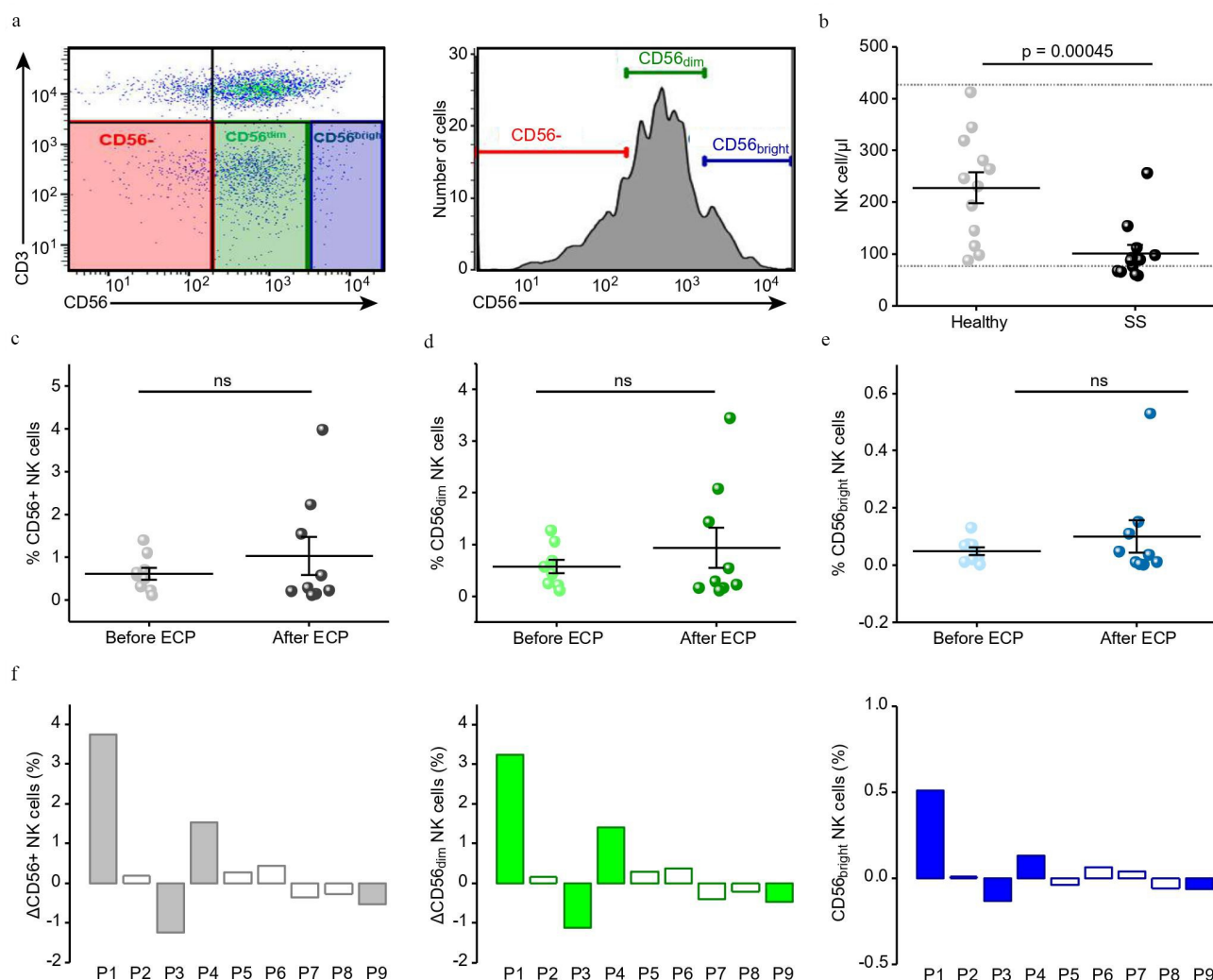


Figure 1. NK cell numbers are reduced in SS compared to healthy and show a tendency of recovery after ECP treatment. (a) Representative FACS plot (left) and histogram (right) illustrating the gating strategy on CD3 (y-axis) and CD56 (x-axis) for CD3⁺/CD56^{dim} and CD3⁺/CD56^{bright} NK cells. (b) Dot plot showing the number of NK cells per μ l whole blood for healthy individuals (Healthy, n = 12) and Sézary patients (SS, n = 12). (c-e) Dot plots depicting the percentage of NK cells in Sézary patients before and after ECP (both groups n = 9), ns = not significant. (c) for total CD56⁺ NK cells (d) for CD56^{dim} NK cells (e) for CD56^{bright} NK cells. (f) Bar plots depicting the relative changes in NK cells for individual Sézary patients before and after ECP treatment, total CD56⁺ NK cells (gray), CD56^{dim} NK cells (green), CD56^{bright} NK cells (blue). Patients with clinical response to treatment are shown as filled bars, those without as empty bars.

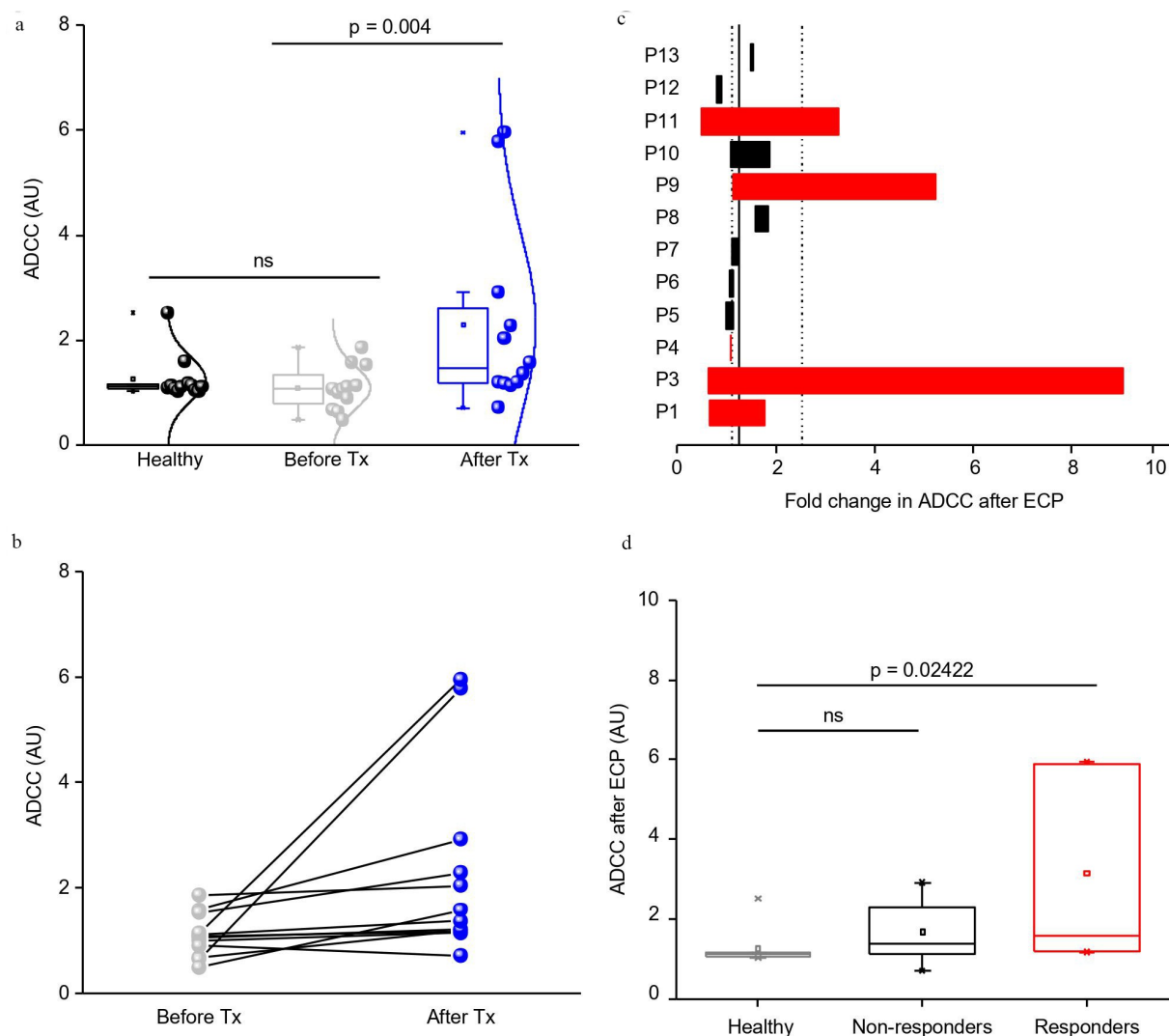


Figure 2. ADCC is enhanced in ECP responders. (a) Boxplots showing the efficacy of antibody-dependent cellular cytotoxicity (ADCC) in arbitrary units (AU) for healthy individuals and Sézary patients before and after ECP treatment (Tx), $n = 12$ for all groups, ns = not significant. A significant increase ($p = .004$) is detected after ECP therapy. (b) Dot plot presenting the same data as in a) for individual Sézary patients, showing great interpatient variability in response to ECP treatment. (c) Bar plots showing fold changes in ADCC of Sézary patients after ECP treatment. ECP clinical responders in red, non-responders in black. (d) Box plots showing the efficacy of ADCC after ECP in arbitrary units (AU) for healthy individuals and Sézary patients split into non-responders and responders. While the difference in non-reponders is not significant (ns), responders show a significant increase ($p = .02422$) in ADCC compared to healthy.

ADCC activity after ECP for non-responders did not differ from the ADCC of healthy individuals, but was statistically significantly enhanced in SS patients responding clinically to ECP treatment ($p = .02422$) (Figure 2d).

Discussion

ECP is an established and widely used first-line therapy for SS; however, predictive factors and biomarkers for monitoring the response to treatment are yet largely unknown. NK cells and their impact and importance for the mode of action of ECP have recently attracted attention, especially in GvHD and organ transplant rejections, which together with SS constitute the three major approved indications for ECP therapy in Switzerland.^{9,18,19} In CTCL, reduced activity of NK cells has been observed, and increased NK cell numbers upon ECP treatment have been reported, raising the hypothesis of

a relative intact immune system being a baseline prediction-factor of response to ECP.^{9,20}

Here we demonstrate that NK-mediated ADCC is selectively enhanced in ECP responders and might serve as a reliable biomarker to objectively monitor response to ECP in patients with SS. These findings are strengthened by observations made in patients with GvHD, where low levels of NK cell activity were reported in non-responders and normal NK-cell activity before therapy has been proposed as a prognostic marker for response.¹⁶ We further observed a general tendency of recovery of diminished NK cell subsets in SS patients on long-term treatment, supporting the casuistically reported increase in NK cell numbers in ECP responders.^{20,21} Further, increased NK cells numbers were also detected in one patient with immune-checkpoint-inhibitor-induced colitis, positively influenced by ECP.²²

Based on our findings and supported by the existing knowledge in the literature, we postulate a vital role of NK cells in

shaping efficacy of ECP in SS patients and support the use of NK-cell-based therapies for tumor immunotherapy. In general, NK cells are seen as a positive factor in the anti-tumor immune response and their involvement in immune surveillance has been repeatedly demonstrated.¹⁷ This study broadens the understanding on the role of NK cells' activity in the context of ECP, although the conclusions are limited by the small number of patients and their differences in treatment duration at the time of sampling ranging between 1.5 and 26 months on ECP. Further, we demonstrate enhancement of ADCC, which is exerted typically by CD56^{dim} subset. However, as we only report trends in the quantitative alterations of these cells, the observed increased efficacy may also result from changes in secreted cytokines and paracrine stimulation of ADCC-mediating cells. Consequently, it will be important for future studies to decipher which NK subpopulation – the immature, poorly cytotoxic but cytokine-producing CD56^{bright} or the mature, weakly cytokine-producing, but cytolytic and thus capable of ADCC, CD56^{dim}CD16⁺ NK cells determine the efficacy of ECP.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ORCID

Christina Fassnacht  <http://orcid.org/0000-0001-5379-2687>

Mirjam Nägeli  <http://orcid.org/0000-0002-2512-2122>

Steve Pascolo  <http://orcid.org/0000-0003-2946-5576>

Malgorzata Bobrowicz  <http://orcid.org/0000-0002-9078-5168>

Emmanuella Guenova  <http://orcid.org/0000-0001-5478-8735>

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